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# Synthesis of (*R*)-Configured 2′-Fluorinated mC, hmC, fC, and caC Phosphoramidites and Oligonucleotides

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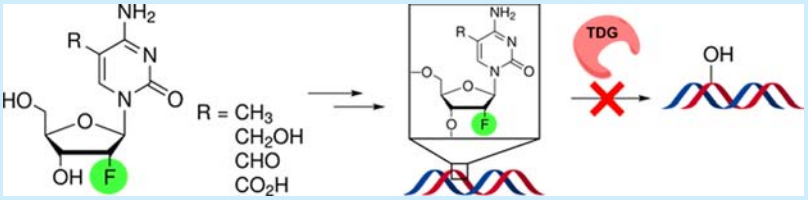
Supporting Information




**ABSTRACT:** Investigation of the function of the new epigenetic bases requires the development of stabilized analogues that are stable during base excision repair (BER). Here we report the synthesis of 2′-(*R*)-fluorinated versions of the phosphoramidites of 5-methylcytosine (mC), 5-hydroxymethylcytosine (hmC), 5-formylcytosine (fC), and 5-carboxycytosine (caC). For oligonucleotides containing 2′-(*R*)-F-fdC, we show that these compounds cannot be cleaved by the main BER enzyme thymine-DNA glycosylase (TDG).

Fluorine is an element that is used in medicinal chemistry to replace H atoms in pharmaceutically active molecules with astonishing effects. Fluorine substitution stabilizes molecules to extend their lifetimes in the bloodstream, and often it increases the affinities of molecules for their biological targets by increasing their lipophilicities.[1] In nucleoside chemistry, for example, fluorination of dC at the 2′ position creates molecules like gemcitabine (**1**), which are used as antimetabolites in modern cancer therapy.[2] The 2′-F substitution has several effects. Most importantly, a 2′-(*R*)-configuration as in 2′-(*R*)-F-dC (**2**) stabilizes the C3′-endo conformation of the ribose sugar so that the base becomes RNA-like.[3] A fluorine at C2′ also blocks the activity of glycosylases, thereby stabilizing the base during base excision repair (BER).[4] We are currently investigating the chemistry that occurs at the nucleoside 2′-deoxycytidine (dC, **3**) that leads to the formation and removal of the methylated and subsequently oxidized epigenetic dC derivatives 5-methyl- (mdC, **4**), 5-hydroxymethyl- (hmdC, **5**), 5-formyl- (fdC, **6**), and 5-carboxy-2′-deoxycytidine (cadC, **7**) (Figure 1).[5] Nucleosides **5**−**7** are products of consecutive enzymatic oxidation of **4** by the action of ten-eleven-translocation enzymes (Tet enzymes), which use molecular oxygen and α-ketoglutarate to perform the oxidation chemistry.[6] Current data suggest that fdC and cadC are removed from the genome by BER via the enzyme thymine-DNA glycosylase (TDG).[5d,7] Other data predict that the bases may undergo some kind of deformylation/decarboxylation reaction, which would convert fdC and cadC directly back into the canonical base dC.[8] In order to distinguish these processes, it is important to have tool molecules that cannot be repaired by BER. This would allow one to decipher chemical processes at fdC and cadC beyond BER. Here we report the synthesis of

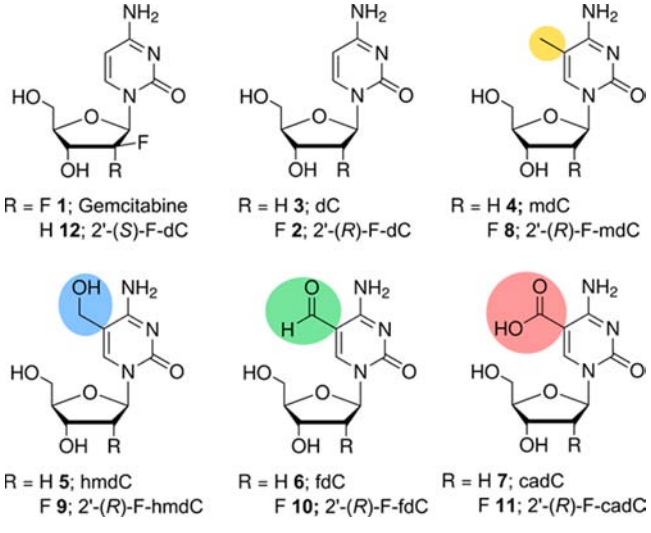



**Figure 1.** Overview of epigenetically relevant nucleosides and 2′-fluoro nucleosides that are important in this context.

the 2′-(*R*)-fluorinated versions of mdC (**8**), hmdC (**9**), fdC (**10**), and cadC (**11**). We have developed phosphoramidite building blocks for the incorporation of these bases into DNA strands, and we show that these nucleosides are indeed stable during BER. With the plan in mind to investigate epigenetic processes directly in the genome of stem cells, we realized that the 2′-*arabino*-configured compound 2′-(*S*)-F-dC (**12**) might be too toxic. Indeed, when we evaluated the toxicity of the *ribo*-

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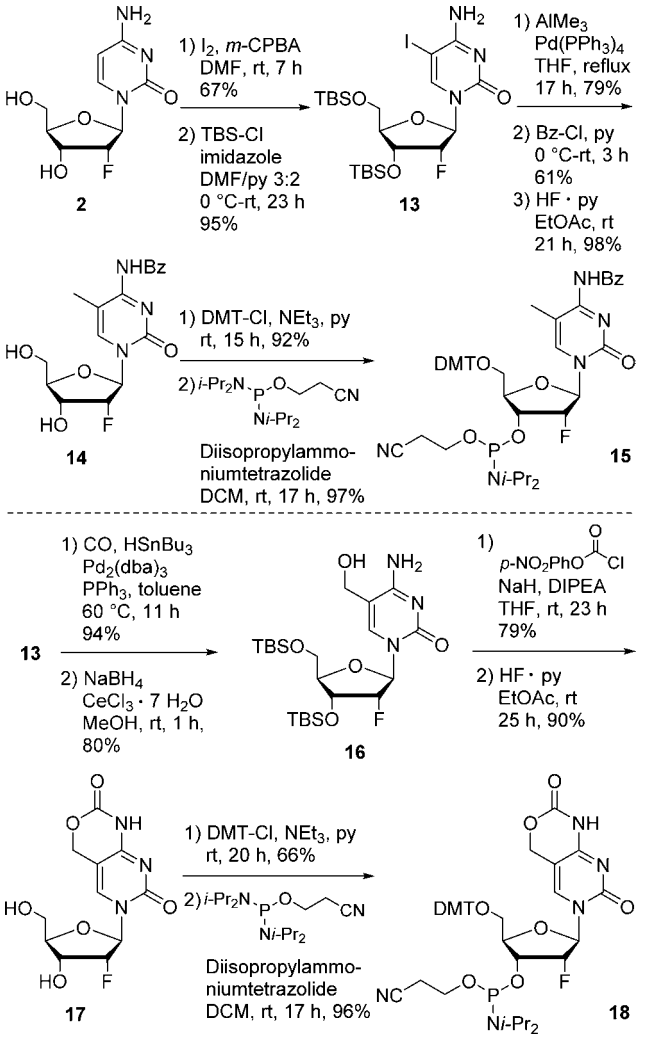
configured compound 2′-(*R*)-F-dC against **12** in stem cells (see the Supporting Information), we noted a strongly reduced toxicity for 2′-(*R*)-F-dC. This is already interesting because it is believed that the 2′-(*S*)-F configuration has a much smaller impact on the overall DNA structure.[3,9] Our stem cell data are, however, in full agreement with toxicity studies in rats and woodchucks showing that feeding of 2′-(*R*)-F-dC at up to 500 mg $kg^{-1}$ $day^{-1}$ is possible without considerable toxicity effects.[10]

For the synthesis of the 2′-(*R*)-F-xdC nucleosides and phosphoramidites **15**, **18**, **21**, and **24** (see Scheme 1), we started with 2′-(*R*)-F-dC (**2**), which was iodinated at C5 with elemental iodine and *m*-CPBA.[11] Subsequent silylation yielded TBS-protected 5-iodo-2′-(*R*)-F-dC **13**. The needed methylation was best carried out under Kumada conditions with trimethylaluminum.[12] This furnished the 2′-(*R*)-F-mdC compound in 79% yield. Notably, the use of other methyl-transferring agents such as MeMgCl resulted in a 1:1 mixture of methylated and dehalogenated products. We believe that the exocyclic amine requires complete deprotonation to avoid a 1,3-proton shift from the exocyclic amine to the Pd-activated C5-position. Further protection with BzCl and silyl deprotection with Olah's reagent furnished 2′-(*R*)-F-mdC derivative **14**, which was converted into the 2′-(*R*)-F-mdC phosphoramidite building block **15** using standard procedures.[13]

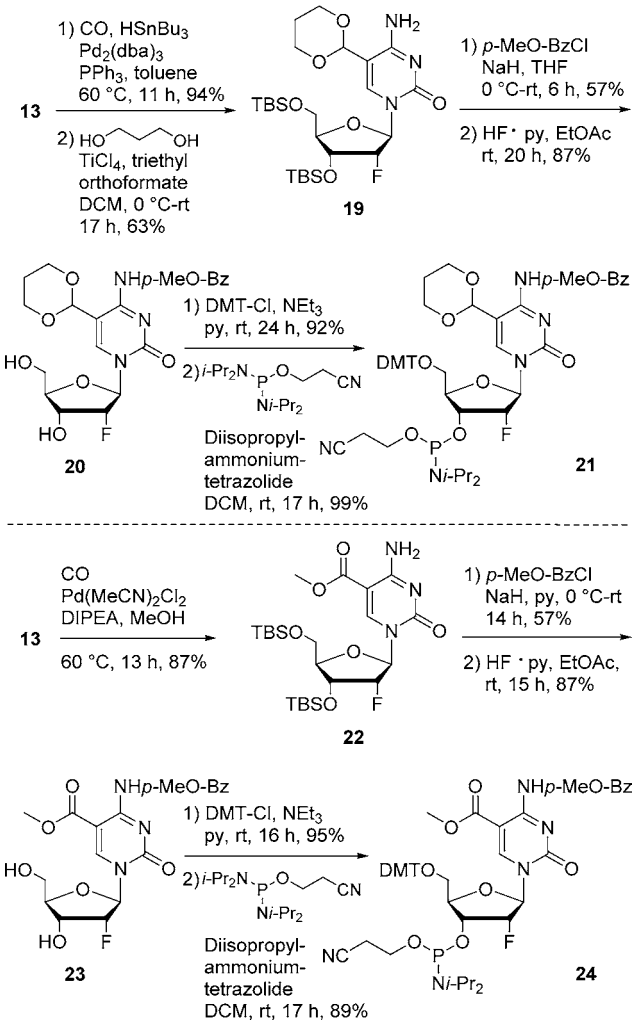
**Scheme 1. Synthesis of 2′-(*R*)-F-mdC and 2′-(*R*)-F-hmdC Phosphoramidite Building Blocks 15 and 18**




For the synthesis of the hmdC phosphoramidite **18**, we started from intermediate **13**. Carbonylative Stille coupling with tributyltin hydride and reduction of the formyl group under Luche conditions yielded 2′-(*R*)-F-hmdC derivative **16**.[14] The exocyclic amine together with the hydroxyl group was protected as a carbamate using *p*-nitrophenyl chloroformate.[15] Efficient conversion required full deprotonation of both functional groups with NaH prior to addition of the protecting reagent. Final silyl deprotection, DMT protection, and synthesis of the hmdC phosphoramidite building block **18** with Bannwarth's reagent furnished the 2′-(*R*)-F-hmC phosphoramidite in high yield (34% over six steps from **13**).

Regarding 2′-(*R*)-F-fdC phosphoramidite building block **21**, we performed a carbonylative Stille coupling reaction of **13** with tributyltin hydride (see Scheme 2). Subsequent masking of the formyl group as a 1,3-dioxane unit with 1,3-propanediol and $TiCl_4$ as the activating Lewis acid provided compound **19**. For the protection of the exocyclic amine, we chose *p*-$MeOC_6H_4COCl$ as recently reported.[16] The electron-pushing methoxy unit strongly enhances the stability of the amine protecting group during solid-phase DNA synthesis, and this is

**Scheme 2. Synthesis of 2′-(*R*)-F-fdC and 2′-(*R*)-F-cadC Phosphoramidite Building Blocks 21 and 24**



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strictly required in order to obtain oligonucleotides in high yields. Again, satisfactory yields were obtained only when the exocyclic amine was deprotonated with NaH prior to addition of *p*-MeOC$_6$H$_4$COCl. Final silyl deprotection yielded **20**, which was converted into 2′-(*R*)-F-fdC phosphoramidite building block **21** using standard procedures.

Starting from intermediate **13**, we next developed the synthesis of the 2′-(*R*)-F-cadC phosphoramidite building block. The synthesis of the methyl ester was achieved using Pd$^0$-mediated CO insertion in methanol.[17] Because of the electron-withdrawing nature of the ester moiety, we decided to use *p*-MeOC$_6$H$_4$COCl for stable protection of the exocyclic amine. Conversion of **23** using standard procedures delivered 2′-(*R*)-F-cadC phosphoramidite building block **24** in just five steps in an overall yield of 36% starting from **13**.

To examine the ability to prepare oligonucleotides containing 2′-(*R*)-F-xdC, we prepared the corresponding **ODN1a–d** (see Figure 2). The modified nucleotides were

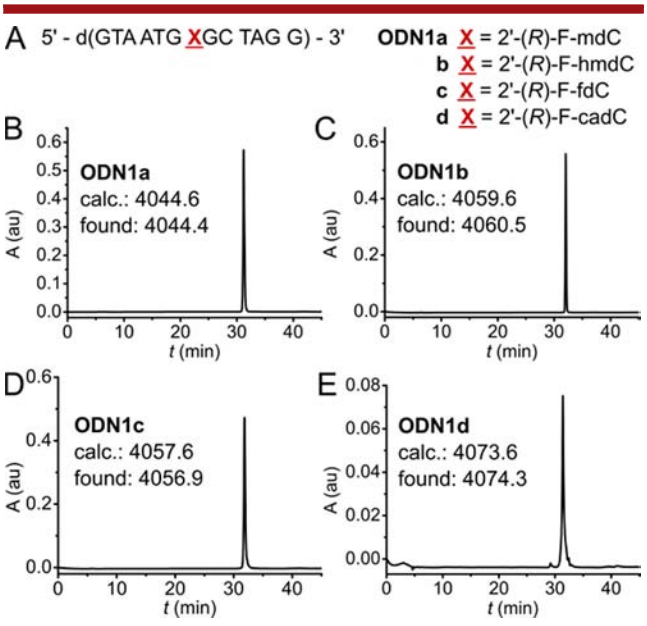


**Figure 2.** (A) Sequence of the synthesized **ODN1a–d** with incorporation of the corresponding 2′-(*R*)-F-xdC phosphoramidite building blocks. (B–D) Reversed-phase HPL chromatograms and MALDI-TOF data for the corresponding purified **ODN1a–d** after basic and, in the case of 2′-(*R*)-F-fdC, acidic cleavage from the resin and deprotection.

placed in a CpG context. The solid-phase syntheses were performed using standard phosphoramidite conditions.[18] For the 2′-(*R*)-F nucleosides, the coupling times were increased from 30 to 180 s to ensure good coupling yields. For deprotection of the oligonucleotides containing 2′-(*R*)-F-mdC and 2′-(*R*)-F-fdC, including cleavage from the solid support, we first treated the solid-phase material with saturated aqueous ammonia solution (18 h, 25–28 °C). Subsequently, the oligonucleotide containing 2′-(*R*)-F-fdC was exposed to aqueous acetic acid (80%) at 20 °C until MALDI-TOF/MS analysis indicated complete hydrolysis of the 1,3-dioxane unit (~6 h). Because of the carbamate and ester units, the oligonucleotides containing 2′-(*R*)-F-hmdC and 2′-(*R*)-F-cadC were deprotected with NaOH (0.4 M in 4:1 methanol/water) for 18 h. This procedure avoided the formation of aminomethyl and amide moieties.[11,17a] Analytical reversed-phase HPLC directly after deprotection showed in all cases just one major product. After purification, the corresponding oligonucleotides were obtained in 20–52% yield and high purity (>95%). MALDI-TOF/MS spectra showed the expected masses, confirming the presence of the 2′-(*R*)-F-xdC bases in the ODNs. In summary, the synthesized 2′-(*R*)-F-xdC phosphoramidite building blocks enabled the synthesis of oligonucleotides containing the corresponding fluorinated nucleosides.

We next started to evaluate the extent to which the 2′-(*R*)-F substitution would affect typical epigenetic processes. First, we wanted to know whether the H-to-F chemical mutation influences the activity of methyltransferases (see Figure 3).

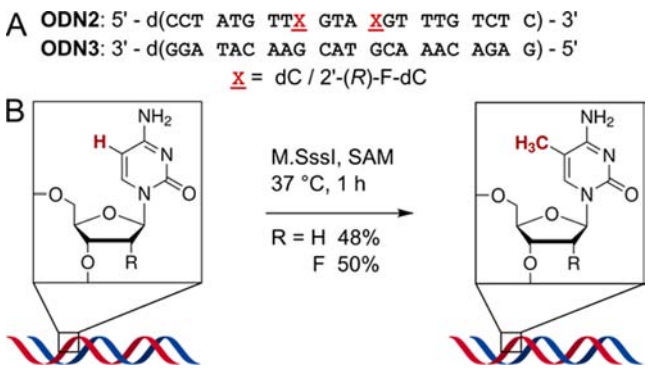


**Figure 3.** (A) Sequences of the synthesized **ODN2** and **ODN3** with incorporation of dC or 2′-(*R*)-F-dC nucleoside. (B) The methylation assay of **ODN2** and **ODN3** with methyltransferase M.SssI showed that the fluoro label in 2′-(*R*)-F-dC has no influence on the level of methylation.

To study this, we synthesized **ODN2** having either dC or 2′-(*R*)-F-dC in a CpG context. After hybridization of **ODN2** with **ODN3**, they were incubated with methyltransferase M.SssI. To determine the level of mdC or 2′-(*R*)-F-mdC, we digested the DNA strands to the nucleoside level and performed UHPLC-MS/MS (QQQ) analysis. As the verification of our hypothesis, we observed methylation of dC (48%) and 2′-(*R*)-F-dC (50%). This demonstrates that the 2′-(*R*)-F substitution does not affect the native behavior of the DNA and that 2′-(*R*)-F-xdC nucleosides are suitable tools for the investigation of the active demethylation beyond base excision repair.

In 2011 and 2012, the groups of Drohat[7a] and Cheng[19] showed that fdC and cadC are excised by human TDG (hTDG). Previously, glycosylase activity was blocked with fluorinated DNA bases (2′-F-(*S*)-cadC, 2′-F-(S/*R*)-dU).[4a,c] In order to determine whether the 2′-(*R*)-F-fdC compounds would block hTDG activity, we synthesized oligonucleotides **ODN4** with either the fdC or F-fdC nucleoside at a central position and hybridized the strands to the complementary oligonucleotide **ODN5**. After hybridization and incubation with hTDG, the DNA strand was treated with piperidine.[7a,19,20] Subsequently, we analyzed the products by HPLC (see Figure 4). As expected, we detected complete strand cleavage for the fdC-containing **ODN4**. However, in the case of the **ODN4** containing 2′-(*R*)-F-fdC, we did not observe any strand cleavage products. Thus, we proved that the 2′-(*R*)-F label indeed inhibits the hTDG activity, blocking BER of fdC.

In summary, we have synthesized 2′-(*R*)-F phosphoramidite building blocks of the epigenetically relevant nucleosides. These building blocks enabled the synthesis of oligonucleotides



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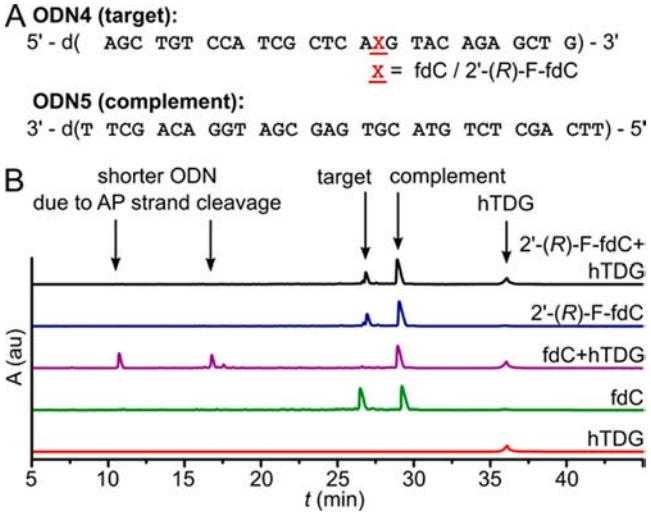


**Figure 4.** (A) Sequence of synthesized **ODN4** and **ODN5** for the hTDG glycosylation assay. (B) Reversed-phase HPL chromatogram of the hTDG glycosylation assay.

containing 2′-(*R*)-F-mdC, 2′-(*R*)-F-hmdC, 2′-(*R*)-F-fdC, and 2′-(*R*)-F-cadC in high yields and quality. Furthermore, we showed that a 2′-(*R*)-F label on fdC blocks the activity of the critical TDG enzyme, thus inhibiting base excision repair of this base. The 2′-(*R*)-F label is consequently the ideal tool for analysis of the epigenetic metabolism beyond base excision.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02110.

Synthesis of all compounds and oligonucleotides and details of the assays (PDF)

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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